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Cytogenetic characterization and genome size of the medicinal plant *Catharanthus roseus* (L.) G. Don

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Abstract

Background and aims *Catharanthus roseus* is a highly valuable medicinal plant producing several terpenoid indole alkaloids (TIAs) with pharmaceutical applications, including the anticancer agents vinblastine and vincristine. Due to the interest in its TIAs, *C. roseus* is one of the most extensively studied medicinal plants and has become a model species for the study of plant secondary metabolism. However, very little is known about the cytogenetics and genome size of this species, in spite of their importance for breeding programmes, TIA genetics and emerging genomic research. Therefore, the present paper provides a karyotype description and fluorescence *in situ* hybridization (FISH) data for *C. roseus*, as well as a rigorous characterization of its genome size.

Methodology The organization of *C. roseus* chromosomes was characterized using several DNA/chromatin staining techniques and FISH of rDNA. Genome size was investigated by flow cytometry using an optimized methodology.

Principal results The *C. roseus* full chromosome complement of $2n = 16$ includes two metacentric, four subtelocentric and two telocentric chromosome pairs, with the presence of a single nucleolus organizer region in chromosome 6. An easy and reliable flow cytometry protocol for nuclear genome analysis of *C. roseus* was optimized, and the C-value of this species was estimated to be $1C = 0.76$ pg, corresponding to 738 Mbp.

Conclusions The organization and size of the *C. roseus* genome were characterized, providing an important basis for future studies of this important medicinal species, including further cytogenetic mapping, genomics, TIA genetics and breeding programmes.

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Introduction

Catharanthus roseus (L.) G. Don (formerly *Vinca rosea* L.) is an important medicinal plant that accumulates in its leaves the dimeric terpenoid indole alkaloids (TIAs) vinblastine and vincristine. These compounds were the first natural anticancer agents to be clinically used and, together with a number of semi-synthetic derivatives, are universally known as the Vinca alkaloids (Sottomayor and Ros Barceló 2006). *Catharanthus roseus* is also the source of two other medicinal TIAs—the antihypertensive ajmalicine and the sedative serpentine.

The great pharmacological importance of the TIAs and their low abundance in the plant (around 0.0005 % of dry weight) has stimulated intense research on the TIA pathway with the aim of manipulating plant metabolism to increase levels of the alkaloids. Consequently, *C. roseus* has become one of the most extensively studied medicinal plants (van der Heijden *et al.* 2004; Verpoorte *et al.* 2007). The biosynthesis of TIAs was shown to be highly complex, involving more than 30 enzymatic steps, and much is already known about the pathway, the enzymes/genes involved and its regulation (Verpoorte *et al.* 2007; Costa *et al.* 2008). Recent genomic/transcriptomic/proteomic/metabolomic approaches have contributed further with much molecular information on *C. roseus* metabolism (Rischer *et al.* 2006; Verpoorte *et al.* 2007; Murata *et al.* 2008). However, there are still many gaps in knowledge about the TIA pathway, its genetics and regulatory mechanisms.

Recent easy access to high-throughput sequencing and the large size of the scientific community interested in *C. roseus* can be expected to generate a wealth of new genome sequence data in the near future. In fact, extensive transcriptomic data for *C. roseus* have just been released (Websites 1 and 2). This emerging genomic research would benefit much from accessibility to detailed cytogenetic data, but current information is very limited in scope and detail—previous publications report only the chromosome number of $2n = 16$ for *C. roseus* (Ma *et al.* 1984; Balamani and Rao 1985; Ge and Li 1989). In fact, the use of detailed karyotypes is instrumental in assigning linkage groups and mapping genes in chromosomes, being essential for the integration of physical and genetic maps towards a full understanding of genome organization (Fransz *et al.* 1998). Detailed karyological information will also help to obtain physical maps to assist breeding programmes, namely aiming at the production of varieties with higher levels of dimeric alkaloids and with resistance to afflicting diseases such as *Pythium* dieback. Other relevant applications may be to help in determining the ancestry of the TIA pathway and to facilitate the understanding of TIA genetics as a whole. On the other hand, flow cytometric techniques are now increasingly being applied to plant cells, enabling rapid and accurate quantification of genome size, and providing complementary useful information (Loureiro *et al.* 2008).

The present paper aims to rectify the above-mentioned shortcomings by providing a detailed karyotype description of *C. roseus* based on several DNA/chromatin staining techniques and fluorescence *in situ* hybridization (FISH) of rDNA. An accurate quantification of genome size is also provided using an optimized flow cytometry protocol developed before for recalcitrant species (Loureiro *et al.* 2007).

Materials and methods

Plant material

Seeds of *C. roseus* (L.) G. Don cv. Little Bright Eye were acquired from AustraHort (Australia). Seeds were germinated on moist filter paper for 2–4 weeks, and seedlings were transferred to pots with compost (COMPO SANA® Universal Potting Soil) all at 25 °C, with a 16 h photoperiod (photosynthetically active radiation 30 μmol m^{-2} s^{-1}). Voucher specimens were deposited at the Herbarium of the Department of Biology of the University of Porto (PO 61912).

Preparation of mitotic spreads

Seeds were treated with 70 % ethanol for 1 min, followed by two washes of 5 min with distilled water. The seeds were germinated on moist filter paper, and 7–10 days later, 10- to 15-mm-long root tips were collected. Approximately 70–100 tips were placed in a microcentrifuge tube containing distilled water and kept at 0–4 °C for 24 h to accumulate cells in metaphase. Water was then removed and root tips were fixed in ethanol : glacial acetic acid, 3 : 1 (v/v), for 2 h at room temperature (RT). Fixed root tips were stored at −20 °C in fresh fixing solution.

Preparation of mitotic spreads was as described by Andras *et al.* (1999), with some modifications. After fixation, root tips were washed twice with distilled water for 5 min. Root apexes (2 mm) were isolated, incubated in 50 μL of 1 M HCl at 37 °C for 25 min, washed for 1 min at RT with 50 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and incubated in 200 μL of an enzyme mixture [4 % (w/v) cellulase Onozuka R10 (Yakult Honsha Co., Hyogo, Japan) and 1 % (w/v) pectolyase (Seishim Pharmaceutical, Tokyo, Japan) in citrate buffer (0.01 M citric acid, 0.01 M sodium citrate, pH 4.6)] for 2 h 30 min at 37 °C. The resulting protoplast suspension was centrifuged at 700 *g* for 3 min at RT, and

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the protoplast pellet was washed twice with 200 μL of distilled water and twice with 200 μL of a fixing solution of methanol : glacial acetic acid, 4 : 1 (v/v). Finally, the protoplasts were resuspended in 70–100 μL of the fixing solution (1 μL per apex), and aliquots (~8 μL) of this suspension were dropped from a height of ~1 cm with a 20 μL micropipette onto the centre of uncoated microscope slides previously washed with 96 % ethanol. Slides were allowed to dry and then incubated at 37 °C for 24 h, prior to observation. For FISH assays, slides were previously coated with 3-aminopropyltriethoxysilane (APES; Sigma-Aldrich, Dorset, UK) and were always freshly prepared.

Silver staining, fluorescence and C-banding

For the silver staining of chromosomes, slides were prepared as above and silver staining was performed as described by Ahmad *et al.* (1999).

For the fluorescence banding of chromosomes with actinomycin D and 4′,6-diamidino-2-phenylindole (AD/DAPI), slides were immersed in McIlvaine's buffer pH 7.0 (0.1 M citric acid plus 0.2 M Na_2HPO_4) for 30 min at RT, and then briefly rinsed with distilled water. A 20 μL volume of 25 μg mL^{-1} AD in McIlvaine's buffer was applied and the slides were incubated in a humid chamber at RT for 15 min. The slides were washed with buffer for 10 min, briefly rinsed with distilled water, and incubated with 20 μL of 1 μg mL^{-1} DAPI in McIlvaine's buffer for 10 min in a humid chamber at RT. Finally, the slides were briefly rinsed with distilled water, mounted in 8 μL of fluorescence mounting medium [McIlvaine's buffer: 87 % glycerol (1 : 1; v/v) and 2.33 % (w/v) DABCO], and kept at 4 °C in the dark for at least 24 h, prior to observation.

For the C-banding of chromosomes, slides were immersed in denaturing solution (0.2 N NaOH in 70 % ethanol) for 5 min at RT, and washed twice with 2 × SSC for 1 and 5 min. Slides were dehydrated for 2 min baths in a graded series of 70, 85 and 100 % ethanol and air-dried. In all, 20 μL of 2 × SSC were applied, overlaid with a coverslip and incubated in a humid chamber at 60 °C for 1 h, followed by a brief rinse with distilled water. For staining, 20 μL of a solution with 2 μg mL^{-1} propidium iodide (PI) and 1 μg mL^{-1} DAPI in McIlvaine's buffer were applied, and the slides were incubated in a humid chamber for 15 min at RT. Finally, the slides were briefly rinsed with distilled water and mounted as described above for AD/DAPI staining.

Morphometric analysis

Four prometaphases at the same condensation state (as determined by the total length of the chromosome complement) were selected and homologues were paired manually and analysed using the program MicroMeasure 3.3 (available at Website 3). Heterochomatin regions highlighted by AD/DAPI staining were also measured. The chromosome type was attributed according to Levan *et al.* (1964).

Labelling of rDNA probe

Heterologous rDNA sequences were used for FISH. Clone pTa71 contains an 8.9 kb rDNA genomic fragment of wheat, consisting of the transcribed and non-transcribed spacer regions of the gene unit 18S-5.8S-26S (Gerlach and Bedbrook 1979), and was kindly supplied by Trude Schwarzacher and Pat Heslop-Harrison (University of Leicester, UK). Plasmid DNA was labelled by nick translation using biotin-14-dATP of the Bionick™ Labeling System (Invitrogen Life Technologies, Paisley, UK). Plasmid DNA (0.5–1 μg) was labelled using 50 μL of labelling reaction (using 5 μL of 10× enzyme mix) for 2 h at 16 °C, and the reaction was stopped with 5 μL of 500 mM EDTA (pH 8.0).

Fluorescence *in situ* hybridization

Chromosomal DNA was denatured by immersing the slides in 0.2 N NaOH in 70 % ethanol for 5 min at RT, followed by two washes with 2 × SSC for 1 and 5 min. The slides were then dehydrated as above and air-dried. The hybridization mixture per slide consisted of 100 ng (~2 ng μL^{-1}) of labelled probe DNA, 1 × Denhardt's solution [0.02 % (w/v) Ficoll, 0.02 % (w/v) polyvinylpyrrolidone (PVP), 0.02 % (w/v) bovine serum albumin], 50 % (v/v) formamide, 2 × SSC and 0.1 % (w/v) SDS. This mixture was denatured by boiling for 5 min, and then immediately quenched on ice for at least 10 min. Hybridization was performed at 37 °C for 48 h in a humid chamber, and the slides were then washed with 2 × SSC for 2 × 5 min at RT, 2 × SSC for 5 min at 52 °C, 2 × SSC for 5 min at RT, and 4 × SSC for 5 min at RT. Labelling was performed at 37 °C for 1 h in a humid chamber with 2 % (v/v) fluorescein–avidin D in 4 × SSC followed by washing for 2 × 5 min with 4 × SSC at RT. The slides were air-dried, counterstained with 2 μg mL^{-1} DAPI for 15 min at RT, rinsed with distilled water, mounted as above, and kept at 4 °C in the dark for at least 24 h prior to observation. Monochromatic images (8 bit) were captured with a digital camera CCD SPOT (Diagnostic Instruments) coupled to a Zeiss Axioskop fluorescence microscope using an immersion objective Zeiss Neofluar ×100. Digital treatment of images was performed with PaintShopPro 6.02 and Fiji (Website 4).

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Flow cytometry analysis

Flow cytometry analysis of *C. roseus* leaf samples was performed as described by Loureiro *et al.* (2007) with some modifications. Young leaf pieces (2nd–3rd pair) with ~50 mg were cut and placed on glass Petri dishes. One millilitre of Woody Plant Buffer [WPB; 0.2 M Tris-HCl, 4 mM $MgCl_2 \cdot 6H_2O$, 2 mM EDTA, 86 mM NaCl, 10 mM sodium metabisulphite, 1 % PVP-10 (w/v), 1 % Triton X-100 (v/v), pH 7.5] was added to the samples, and the leaf pieces were intensively chopped using a razor blade. The resulting homogenate was filtered through a 50 μm nylon mesh, and 50 μL of RNase (1 mg mL^{-1}; Sigma, St Louis, MO, USA) and 50 μL of PI (1 mg mL^{-1}; Fluka, Buchs, Switzerland) were added to the filtrate. The mixture was incubated for 5 min at RT and loaded onto the flow cytometer (Epics XL with a 488 nm laser; Beckman Coulter, High Wycombe, UK) to determine the ploidy level and genome size. As the sample had a small genome size, we used *Bellis perennis* as a secondary internal reference standard, as also used recently by others (Leong-Škorničková *et al.* 2007; Kolár *et al.* 2009). *Bellis perennis* has a 2C-value of 3.65 pg DNA, which we routinely confirmed using as an internal standard *Pisum sativum* cultivar 'Ctirad' obtained from certified seeds and with a 2C-value of 9.09 pg DNA (Doležel and Greilhuber 2010). Genome sizes were calculated according to the following formula: DNA content (pg/2C) = (G0-G1 average peak × DNA content of internal standard)/(G0-G1 average peak of internal standard). Mass values were converted into base-pair numbers using the factor 1 pg = 978 Mbp (Doležel *et al.* 2003).

Results

The *C. roseus* karyotype

Staining of chromosomes with AD/DAPI permitted an unambiguous morphological distinction of all chromosomes at metaphase and especially at prometaphase (Fig. 1D and G), due to the appearance of bands with strong fluorescence usually attributed to heterochromatin. Therefore, this technique was chosen for the construction of the karyotype and respective ideogram as represented in Fig. 1G and H. The *C. roseus* karyotype includes $2n = 16$ chromosomes, with two metacentric, four subtelocentric and two telocentric chromosome pairs. The total length of the chromosome complement is around 51 μm at prometaphase, and an overview of all the morphometric data is given in Table 1.

Most chromosomes are highly asymmetrical, either subtelocentric or telocentric, with centromere indexes below 25 %. No submetacentric chromosome was found. Chromosome 6 may be considered metacentric and chromosome 8, although it looks telocentric at the prometaphases used for the morphometric study (Fig. 1G), is indeed metacentric. In fact, one of the arms of chromosome 8 is usually uncondensed and/or not preserved at prometaphase, and is only totally visible at metaphase (Fig. 1F). In this later phase, it was possible to identify this chromosome and conclude that it is metacentric—measurements were performed and inferred for prometaphase (Table 1). Chromosomes 8 and 3 show constrictions in their long arms.

Banding techniques and FISH

The chromosome complement of *C. roseus* presents a single active nucleolar organizing region (NOR). In fact, examination of nuclei by silver staining (AgS) revealed the presence of a single nucleolus in interphase and prophase nuclei (Fig. 1A), and prometaphases showed either a single pair of chromosomes attached to the nucleolus (Fig. 1B) or a single pair of Ag-NOR dots located on the short arm of chromosome 6 (Fig. 1C). Likewise, C-banding/PI/DAPI staining strongly highlighted in red the same two regions of chromosome 6 (Fig. 1E), and FISH for rDNA (pTa71 probe—18S-5.8S-26S from wheat) resulted in conspicuous labelling of the same regions (Fig. 1F), indicating the presence of a single 18S-5.8S-26S locus.

Staining of chromosomes with AD/DAPI enabled identification of heterochromatin regions in prometaphase chromosomes, which are essentially pericentromeric and associated to the NOR site. Measurement of those regions indicated that heterochromatin accounts for about 20 % of the total length of the chromosome complement.

Flow cytometry analysis of *C. roseus* nuclei

Flow cytometry analysis of nuclei from *C. roseus* leaves produced cytograms and linear-FL histograms (Fig. 2) with coefficient of variation (CV) values for the G0–G1 peak around 3 %, which ensures the reliability of this protocol. The measurements performed thus enabled reliable estimations of DNA index and relative nuclear DNA content shown in Table 2. Figure 2 shows a representative histogram of particle counts per relative fluorescence intensity, where peak 1 can be clearly assigned to 2C level values of *C. roseus* nuclei, corresponding to the G0-G1 stage, peak 2 can be assigned to the G2 stage and is almost undetectable as expected, and peak 3 belongs to the G0-G1 peak of the internal reference standard *B. perennis* with 2C = 3.65 pg. The corresponding cytogram is shown in the inset of Fig. 2, indicating the robustness of results. The determination of the nuclear DNA content of *C. roseus* in absolute units gave a highly

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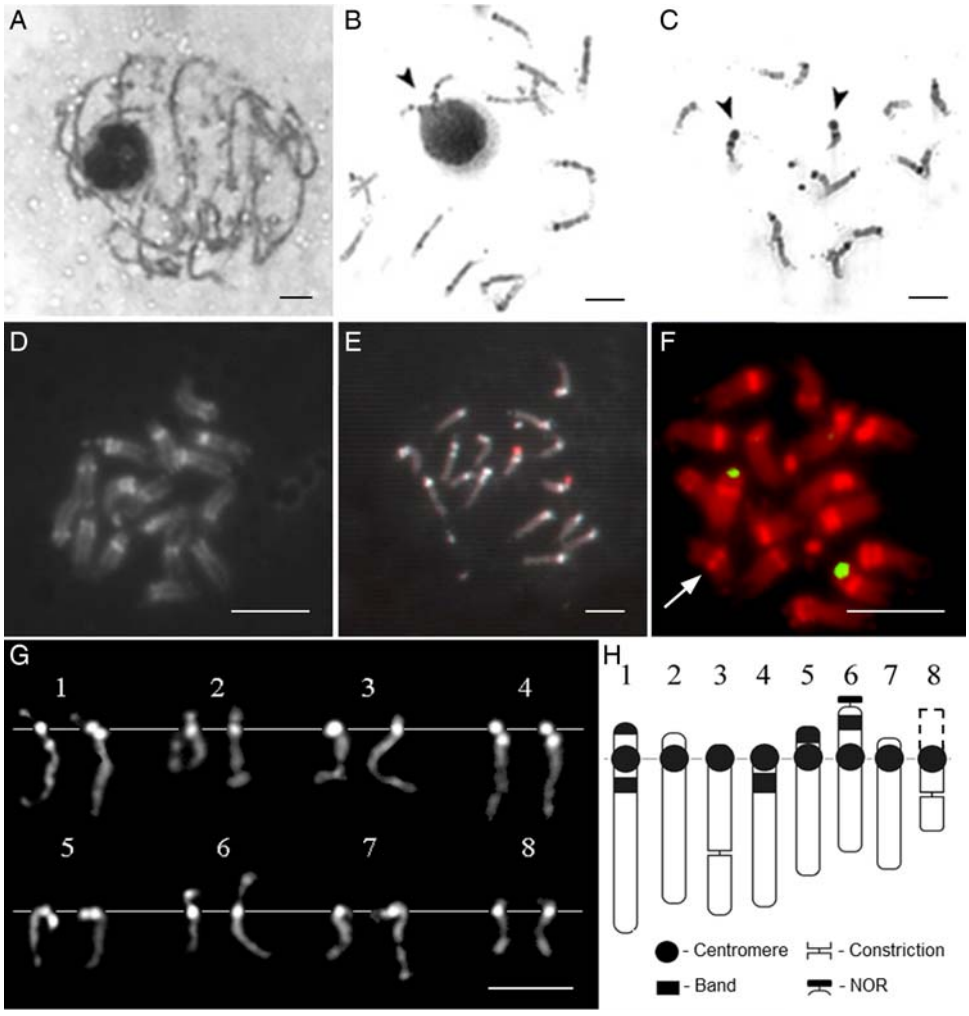

Fig. 1 Cytogenetic analyses of *C. roseus* on mitotic spreads prepared from root tip cells. (A–C) Mitotic spreads of *C. roseus* stained with silver nitrate. (A) Prophase revealing a single nucleolus. (B) Prometaphase revealing a single pair of homologous chromosomes associated to the nucleolus. (C) Prometaphase with an already disorganized nucleolus and with the NORs restricted to a single chromosome pair. (D) Fluorescent staining with AD and DAPI of a prometaphase. (E) C-banding and staining with PI and DAPI of a prometaphase, highlighting the NORs in red. (F) Fluorescence *in situ* hybridization of rDNA of a metaphase. The probe (pTa71) was labelled with biotin-14-dATP and detected with fluorescein-avidin D, the chromosomes were counterstained with DAPI and the NORs are highlighted in green. Arrow indicates chromosome 8. (G) *Catharanthus roseus* karyotype. Chromosomes are aligned by the centromeric region and are ordered from 1 to 8, from the larger to the smaller, according to the morphometric analysis. (H) Ideogram of *C. roseus* chromosomes. Centromeres, constrictions, bands and NORs are depicted in the picture; the small arm of chromosome 8 is represented by a dashed line since it is usually not visible. Scale bars = 5 μm.

reproducible value of 1.51 pg/2C (SD = 0.01). This value was maintained for *C. roseus* plants from other origins, namely for *in vitro* regenerated plants (data not shown).

Discussion

Currently, the development of omic approaches and their growing convenience has driven attention away from the investigation of the organism itself. However, the knowledge accumulated by those approaches will only fully make sense when anchored and integrated to the whole organism picture, including its cytogenetic characterization, especially in what concerns genome sequence information. Although *C. roseus* is an extensively studied plant at the biochemical and molecular levels, very little was known hitherto about its chromosome organization. Previous publications report only the chromosome number of $2n = 16$ (Ma *et al.* 1984;

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Table 1 Morphometric data of the eight chromosome pairs of the *C. roseus* karyotype measured at prometaphase[a]

Chromosome	Average length (μm)	Relative length	Short arm (μm)	Long arm (μm)	Type[b]	Centromere index[c]
1	8.627 ± 0.466	0.17	1.478 ± 0.353	7.149 ± 0.560	st	0.172 ± 0.040
2	7.004 ± 1.335	0.14	1.017 ± 0.236	5.987 ± 1.265	st	0.147 ± 0.038
3	7.002 ± 1.125	0.14	0.518 ± 0.082	6.484 ± 1.138	t	0.076 ± 0.018
4	6.638 ± 0.727	0.13	0.410 ± 0.060	6.228 ± 0.684	t	0.062 ± 0.006
5	6.117 ± 0.504	0.12	1.242 ± 0.185	4.875 ± 0.492	st	0.204 ± 0.030
6	5.963 ± 1.033	0.12	2.246 ± 0.325	3.722 ± 0.808	m	0.380 ± 0.040
7	5.377 ± 0.860	0.10	0.799 ± 0.089	4.578 ± 0.836	st	0.151 ± 0.024
8	4.784[d]	0.09	1.805[d]	2.979 ± 0.541	m	0.377[d]
Total length	51.512	–	–	–	–	–

[a]Values are means of measurements performed in four prometaphases in which the full chromosome complement was visible with high quality ± standard deviations.
[b]Chromosome types were classified according to Levan *et al.* (1964): m, metacentric; sm, submetacentric; st, subtelocentric; t, telocentric.
[c]Length of short arm/total length of chromosome.
[d]Values were estimated for prometaphase based on measurements performed at metaphase.

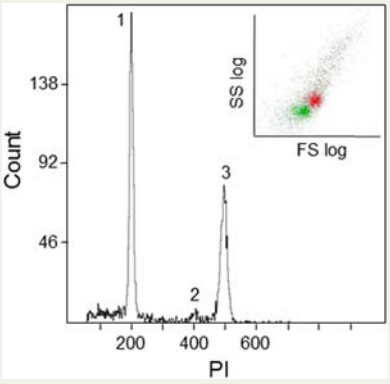

Fig. 2 **Representative histogram of particle counts per relative fluorescence intensity (PI) obtained after simultaneous analysis of nuclei isolated from *C. roseus* leaves and from the internal reference standard *B. perennis* (2C = 3.65 pg DNA).** Three peaks were observed: peak 1—nuclei at G0-G1 phase of *C. roseus*; peak 2—nuclei at G2 phase of *C. roseus*; peak 3—nuclei at G0-G1 phase of *B. perennis*. The G2 phase of *B. perennis* was out of the detection range. The inset represents the forward scatter log (FS log) vs. side scatter log (SS log), where a gating region was defined to exclude doublets and unidentified particles.

Balamani and Rao 1985; Ge and Li 1989), but no morphological description of the chromosomes had been performed. Here, a detailed characterization of *C. roseus* chromosomes was accomplished, including centromere indexes, chromosome lengths, distribution of heterochromatic regions and NOR localization.

The chromosome complement of $2n = 16$ observed confirmed previous reports and is within normal values for Apocynaceae [$x = 8–12(+)$], although in the low border, since the predominant number is $x = 11$ (Albers and Meve 2001). The karyotype and respective ideogram are represented in Fig. 1G and H, and include two metacentric, four subtelocentric and two telocentric chromosome pairs. Many of the chromosomes have a similar centromere localization, which initially made it difficult to differentiate them, but staining with AD/DAPI was found to be a very efficient method for chromosome characterization, producing a high number of morphological marks that enabled full distinction of the eight chromosome pairs. This was particularly true for prometaphase rather than metaphase chromosomes, and therefore prometaphase was used for karyotype characterization as performed by other authors (Iannuzzi 1996).

All techniques used, namely AgS, C-banding/PI/DAPI, AD/DAPI and FISH of rDNA, converged for the presence of a single NOR localized in the small arm of chromosome 6. The localization and number of NOR per nucleus may vary from species to species or be conserved, and may, for example, reflect the occurrence of hybridization or differences in ploidy (Lim *et al.* 2000). The presence of only one nucleolus and one pair of NOR in *C. roseus* may thus be considered normal and is in line with a previous report from electron microscopy studies of this species (Cousin 1980).

In this work, the C-value of *C. roseus* was also estimated through flow cytometry analysis of PI-stained mesophyll nuclei. Our data indicate that *C. roseus* has a

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Table 2 Nuclear DNA content[a] of *C. roseus*

Plant material	DNA index		Nuclear DNA content (pg/2C)		1C[b] (Mbp)	CV (%)[c]	*N*[d]
	Mean	SD	Mean	SD			
Nuclei of leaves	0.413	0.002	1.51	0.01	738.39	3.35	4

[a]The nuclear DNA content was estimated relative to the internal reference standard *B. perennis*.
[b]1 pg DNA = 978 Mbp (Doležel *et al.* 2003).
[c]CV is a mean of CVs of the G0–G1 peak obtained in different measurements.
[d]Number of plants used.

Table 3 DNA amount and molecular size of each *C. roseus* chromosome estimated from the morphometric cytogenetic data and the C-value measured by flow cytometry

Chromosome	Pg	Mbp[a]
1	0.127	124
2	0.103	100
3	0.103	100
4	0.097	95
5	0.090	88
6	0.087	85
7	0.079	77
8	0.070	69
Total (1C)	0.755	738

[a]1 pg DNA = 978 Mbp (Doležel *et al.* 2003).

genome of 1C = 0.76 pg, comprising around 738 Mbp (Table 2). This value, combined with the cytogenetic data, allowed us to estimate the base-pair sizes of each chromosome as detailed in Table 3. The C-value obtained of 1C = 0.76 pg, although similar to the 1C = 0.7 pg described by Zonneveld *et al.* (2005), is very different to the 2.43 pg previously reported by Galbraith *et al.* (1983), which is the value referenced in the Plant DNA C-values Database (Bennett and Leitch 2010). The small differences between our value and that reported by Zonneveld *et al.* (2005) may be attributed to different methodologies: we used an improved buffer to prevent the distorting effects of certain cellular compounds and we incubated the nuclei for only 5 min in PI, as this was demonstrated to be more efficient than longer periods (Loureiro *et al.* 2006, 2007). Moreover, Zonneveld *et al.* (2005) used *Hordeum vulgare* 'Sultan' with 2C = 10 pg DNA as standard, while we used *B. perennis* with 2C = 3.65 pg DNA, which has a much smaller genome, as recommended in analyses of samples with small genome sizes (Leong-Škornicková *et al.* 2007). The conditions described here, in particular the use of WPB, were developed for plants rich in certain secondary metabolites, and resulted in highly accurate data [low % CV (coefficient of variation)] for recalcitrant plants such as *Vitis vinifera, Citrus sinensis, Quercus robur, Olea europaea, Ilex aquifolium* and *Prunus domestica*, among others (Loureiro *et al.* 2007). Indeed, we have observed that some secondary metabolites, especially phenolic compounds in woody species, greatly influence the cytograms obtained using PI staining, in a manner highly dependent on the buffer used. That influence is visible in the cytograms as a typical shift of the events called the 'tannic acid effect' due to the studies demonstrating the occurrence of this effect in the presence of tannic acid (Loureiro *et al.* 2006). Measurements performed with the buffer used by Galbraith *et al.* (1983) for the determination of a C-value of 2.43 for *C. roseus* were shown to be particularly affected by the 'tannic acid effect' (Loureiro *et al.* 2006), and may be one of the causes of the difference in the C-value estimated for *C. roseus* and the high CV reported by Galbraith *et al.* (1983) (9.7 %, much higher than the 3 % reported here). The CV is very important in flow cytometry studies and some authors (Marie and Brown 1993; Galbraith *et al.* 2002) consider it an elementary criterion that reflects the quality of the applied methodology. Marie and Brown (1993) suggested a range of 1–2 % for top-quality analysis in plant cells and 3 % as a routine value. On the other hand, Galbraith *et al.* (2002) suggested a CV of <5 % as the acceptance criterion. But possibly even more relevant as an error source was the use by Galbraith *et al.* (1983) of chicken red blood cells as a reference standard, and the use of mithramycin, which is a non-stoichiometric fluorochrome and therefore inadequate for DNA genome size determinations.

The 1C value of 0.76 pg (~738 Mbp) determined here for *C. roseus* indicates a genome that is roughly six times bigger than the Arabidopsis genome (The Arabidopsis Genome Initiative 2000) and nearly two times bigger than the rice genome (Goff *et al.* 2002; Yu *et al.*

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2002). However, it is well within the range of other plants whose genomes have recently been sequenced, such as sorghum (~730 Mbp) or pigeon pea (~833 Mbp), and indeed much smaller than the now sequenced 2.3 Gbp of the maize genome (Paterson *et al.* 2009; Schnable *et al.* 2009; Varshney *et al.* 2011). To put the *C. roseus* genome size into a broader perspective, the Plant DNA C-values Database (Bennett and Leitch 2010) now indexes 6287 plant species with a 1C mean of 5.94 and comprising values ranging from 0.06 to 152.2. In fact, Leitch *et al.* (2010) report a mode and median genome size for angiosperms of 0.6 and 2.6, which make *C. roseus* genome size a quite common C-value among higher plants.

Conclusions and forward look

In conclusion, we provide here the first detailed description of the organization of the *C. roseus* chromosome complement, and a careful estimation of the C-value of this species. This knowledge will be of importance for the systematization of emerging genome sequence information, and research aimed at investigating the ancestry of the TIA pathway and its genetic characterization. Moreover, this work may be the basis for physical mapping assisting in breeding programmes.

We also provide an improved flow cytometry protocol (short incubation with PI and a buffer designed for recalcitrant woody species) for the easy and reliable evaluation of the C-value of *C. roseus* DNA, which may be used, namely, to monitor ploidy stability throughout regeneration procedures, particularly important when performing the production of genetically manipulated plants for TIA improvement.

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Contributions by the authors

G.G performed all the cytogenetic assays. Authors L.C, H.O and P.D performed the cytometry studies and P.D was also involved in the planning of research and writing of the manuscript. C.S supervised the cytometry studies. M.S was involved in the planning and supervision of all the experimental work, and in writing the manuscript.

Conflicts of interest statement

None declared.

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Appendix

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